PREDOMINANCE OF BACTERIAL SPECIES IN STEADY AND NON-STEADY STATE SYSTEMS

By

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CHAPTER I

INTRODUCTION

Microorganisms play a key role in the treatment of waste water. The bacteria, in general, are mostly responsible for the reduction of the energy level of the waste and, hence, the reduction of the pollutional power of the waste. The interaction of these bacteria brings about the predominance of a few species and the repression of the rest in accordance with the enrichment culture principle which is applicable to many wastes. The predominating organisms are the ones chiefly responsible for the reduction of the energy level of the wastes in various treatment processes and in the receiving body of water. Therefore, if a better understanding of the bacterial interaction can be obtained, better treatment plant design may ensue.

Literature Review

Fawcett (1) expressed the need for studying mixed cultures. He states that the results of a mixture of two or more organisms may be more significant than the effects of pure cultures studies individually.

Sanborn (2) found that the decomposition of cellulose by Cellulomonas folia was aided by other organisms in the same culture. The cellulose degradation was more complete when the other organisms were present to furnish some essential compounds to the Cellulomonas folia.

The food industry has used microorganisms in association for many years. The processing of sauerkraut, green olives, and cheeses are examples of products of microbes in association. Sherman and Shaw (3) have shown that by combining Streptococcus lacticus or Lactobacillus casei with Bacterium acidipropionici, a much increased rate of fermentation of lactose to propionic acid is effected. Imamura and Tsugo (4, 5) found that Streptococcus lactis and Penicillium roqueforti act symbiotically in the ripening of roquefort cheese. The Penicillium roqueforti slowly consumes lactose as compared with the consumption of glucose and galactose, the two monosaccharide constituents of lactose. However, when the two organisms are mixed, consumption of lactose is greatly increased. Peterson, Black, and Gunderson (6, 7, 8, 9, 10) have recently studied the growth of staphylococci in mixed cultures. In one study (6) staphylococci were suppressed by naturally occurring saprophytic species in the precooked frozen foods. The study was made on these precooked frozen foods during defrost. The authors state that: "Apparently the

greater the saprophytic population, the greater the protection against staphyloccal growth through antagonism, competition for nutrients, and modification of the environment to conditions less favorable to staphylococcal growth." These results are in sharp contrast to the results of pure culture studies of staphylococci (7).

Mickelson and Flippin (11) found a strain of Escherichia coli which would eliminate Salmonellae from egg whites.

Rahn (12) found that Pseudomonas aeruginosa inhibited Staphylococcus and typhoid organisms.

Miller (13) states that, "With the exception of predation, grazing, and parasitism, one individual affects the other through the environment and not directly from individual to individual." This effect can be an antagonistic one. Jeney (14) believed that the antagonistic effect of one microorganism on another may be due to the fact that one contains exclusively D-amino acids and the other exclusively L-amino acids. His experimental results have proven a definite antibiotic effect of D-glutamic acid, D-lysine, and D-arginine. Savage and Florey (15) stated that no proof could be obtained from their own experiments that bacteria can be induced to produce an antibiotic for any selected species. Charlton (16) concluded that antagonism between Aerobacter aerogenes and Bacillus subtilis was present on a nutritional basis and not

due to antibiotic production, crowding, or direct contact between organisms. He also concluded that the antagonism was due to competition for gaseous nutrients in the medium.

Synergistic and symbiotic effects may also be brought about by the effects of one organism on another through the environment. Waksman and Lomanitz (17) found that ammonia could be formed from proteins by using a mixed culture of Bacterium cereus and Bacterium fluorescens. Bacterium cereus degrades the protein to amino acids and Bacterium fluorescens forms ammonia from these. Waksman and Hutchings (18) found by using a potent cellulose degrading fungus and a bacteria which could not utilize cellulose, that the rate of cellulose decomposition was faster than that of the fungus alone. The reason given by the authors was that the more easily available carbohydrates were utilized by the bacteria, thus, for the fungus to survive, it had to degrade more cellulose. Nurmikko (19) found that the lactic acid bacteria he studied could grow with growth factors omitted from the medium, provided that the other organisms present could synthesize these growth factors. Borina (20) found that Serratia marcescens hastened the fermentation and increased its intensity when grown in association with Clostridium acetobutylicum on cereal flour. The subject of the study was the effect of Serratia marcescens on the course of acetone-butanol fermentation by

Clostridium acetobutylicum.

The environment of microorganisms also affects the population dynamics. Fawcett (1) found that changes in temperature could alter the predominance of organisms in a culture. Gibson (21) noted microbial population changes when the pH of the rumen of sheep was suddenly lowered by an abrupt change in the diet from hay to grain. Protozoa were killed and gram positive organisms increased.

Gaudy (23), using oxygen uptake as the major criterion, found that a mixture of organisms was generally more effective than pure cultures in removing substrates from the environment. He used four organisms in all possible combinations and also used the following substrates: glucose, glucose and nutrient broth, fructose, sucrose, and maltose. From his work one could conclude that pure culture studies of organisms found to predominate in any waste treatment process would lead one to erroneous results as to the rate of purification of the waste. Even though some indication as to the rate of substrate removal could be obtained, no concrete facts could be correctly utilized in the design of the treatment facilities.

However, this was exploratory work designed to give an insight into bacterial predominance changes during waste water purification. Indeed, it would seem that a considerable amount of such basic research will be needed before extrapolation

to a full scale treatment plant will be possible. One of the great needs of the water pollution control field is for more fundamental understanding of interaction of bacterial species. It will be necessary to do research simply to find out if an understanding of predominance changes in a closely controlled model system can be transposed to the highly complex situation which exists in the natural invironment. These studies were conducted solely in batch systems. In many respects, the present study, which includes both batch and continuous flow investigations, represents the desirable and logical extension of Gaudy's studies.

One problem of activated sludge treatment plants is known as sludge bulking. This problem can be caused by the predominance of filamentous organisms. These organisms prevent the sludge from settling properly because of their filamentous structure. Many investigations into the causes of bulking are found in the literature, but to date the full story of bulking sludge has not been unfolded. Smit (23) has shown that sludge bulking occurs when as much as 1000 p.p.m. of glucose is added to sewage. He points out, however, that sludge bulking occurs in sewage treatment plants where there is no appreciable amount of sugar in the sewage. Ruchhoft (24) isolated an organism which was the cause of sludge bulking at the Des Plaines River activated sludge plant. Ruchhoft

did not identify the filamentous organism he isolated as Sphaerotilus, but the organism resembled Sphaerotilus with respect to its effect on bulking. Heukelekian and Ingols (25) concluded that "addition of nitrates was ineffective in preventing bulking of sludge with sugar but quite effective in preventing bulking of sludge with sewage." From this study one may surmise that the presence of substantial quantities of carbohydrates is not essential to sludge bulking. Ingols and Heukelekian (26) found that sludge bulking would occur when they fed the sludge pure organic compounds and used a limited amount of oxygen. They were able to eliminate bulking when nitrates and either calcium butyrate or peptone were used in combination.

In all of the preceding studies presented, the problem of bulking sludge is approached from the standpoint of type of waste and amount of aeration. No investigations were done on the possible species interaction as a cause of the predominance of the filamentous organisms. As has been shown (26), the addition of nitrates sometimes eliminates bulking. This additional nitrogen in some way allows the other organisms to suppress the filamentous forms in some wastes. Only a basic study of species interaction in mixed culture can unveil the mechanism of action of the nitrates on predominance.

McKinney (27) has stated another problem encountered in

activated sludge treatment which is caused by predominance. When raw sewage enters the aeration tank of the activated sludge process, a primary group of organisms are more efficient in their utilization of this organic matter and, therefore, predominate. When the raw organic matter is utilized as the mixture flows through the tank, a secondary group of microorganisms predominates due to the change of the type of available nutrients. When the sludge is returned to the beginning of the tank, the primary group requires a long aeration period to again predominate and, thus, effect rapid stabilization of the waste. The long aeration period makes the process vulnerable to hydraulic and quantitative shock loads.

To illustrate the change in predominance, the work of Jasewicz and Porges (28) may be cited. By using dairy wastes in a batch type system, they found that 74 per cent of the bacteria in the assimilative phase were either of the genus Bacillus or Bacterium, while only 8 per cent of Bacillus and Bacterium were found in the endogenous phase. In the endogenous phase some 42 per cent of the bacteria were Alcaligenes and Pseudomonas and 48 per cent were Flavobacterium or Micrococcus.

The preceding has been a brief review and discussion of the literature dealing most closely with the subject of this thesis. Since most wastes have an extremely heterogeneous

population, the problem of understanding the effects of environment and association of organisms is complex and is a difficult one to study. The author feels that by elimination of the uncontrollable variables introduced by the use of the heterogeneous system, some insight into the question of causation of predominance which would aid in understanding waste water purification processes may be obtained.

Some of the variables which can be made nearly constant are: number of species which have a part in the interaction of the heterogeneous population, pH, temperature, concentration and type of waste, dissolved oxygen concentration, degree of agitation, and rate of introduction of waste to the system. In the studies presented here all of the above factors were maintained as constant as possible with the exception of the rate of introduction of the synthetic waste to the system. This parameter was varied in a known manner to control the growth rate in the continuous flow system herein studied.

Kinetic Considerations

The studies undertaken, dealing with the factors causing predominance, were generally of two types. One type was a study of the growth kinetics of pure and mixed cultures in batch or discontinuous systems. In these studies no environmental changes were imposed on the organisms other than the

ones the bacteria themselves created. In the other type study, the bacteria were grown in pure and mixed cultures and were maintained under continuous flow steady state conditions. This type of study could eliminate predominance of one organism due to the build up of intermediate metabolites or end products and allow for interaction based on the ability to compete for growth factors. It was realized that continuous flow experiments would not completely eliminate the possible interaction of intermediate metabolites or end products on the competition.

In order to use the mathematical model employed in this study, a basic understanding of the concepts of bacterial growth kenetics is necessary. Concepts which govern batch systems were aptly formulated by Monod (29), who found a relationship between the growth rate of the organism and the concentration of the limiting growth metabolite. Based upon his experimental results, he presented the following equation:

$$\mu = \mu_m s/(s + K_s) \quad . \quad . \quad . \quad . \quad . \quad (1)$$

where μ is the growth rate, μ_m is the maximum growth rate, s is the concentration of the limiting growth substance, and K_s is the concentration of s which gives one-half the maximum growth rate (see Figure 1). Monod (30, 31) presented data on the growth of Escherichia coli and Mycobacterium tuberculosis which fit the equation very well.

The rate of increase of organisms is proportional to the

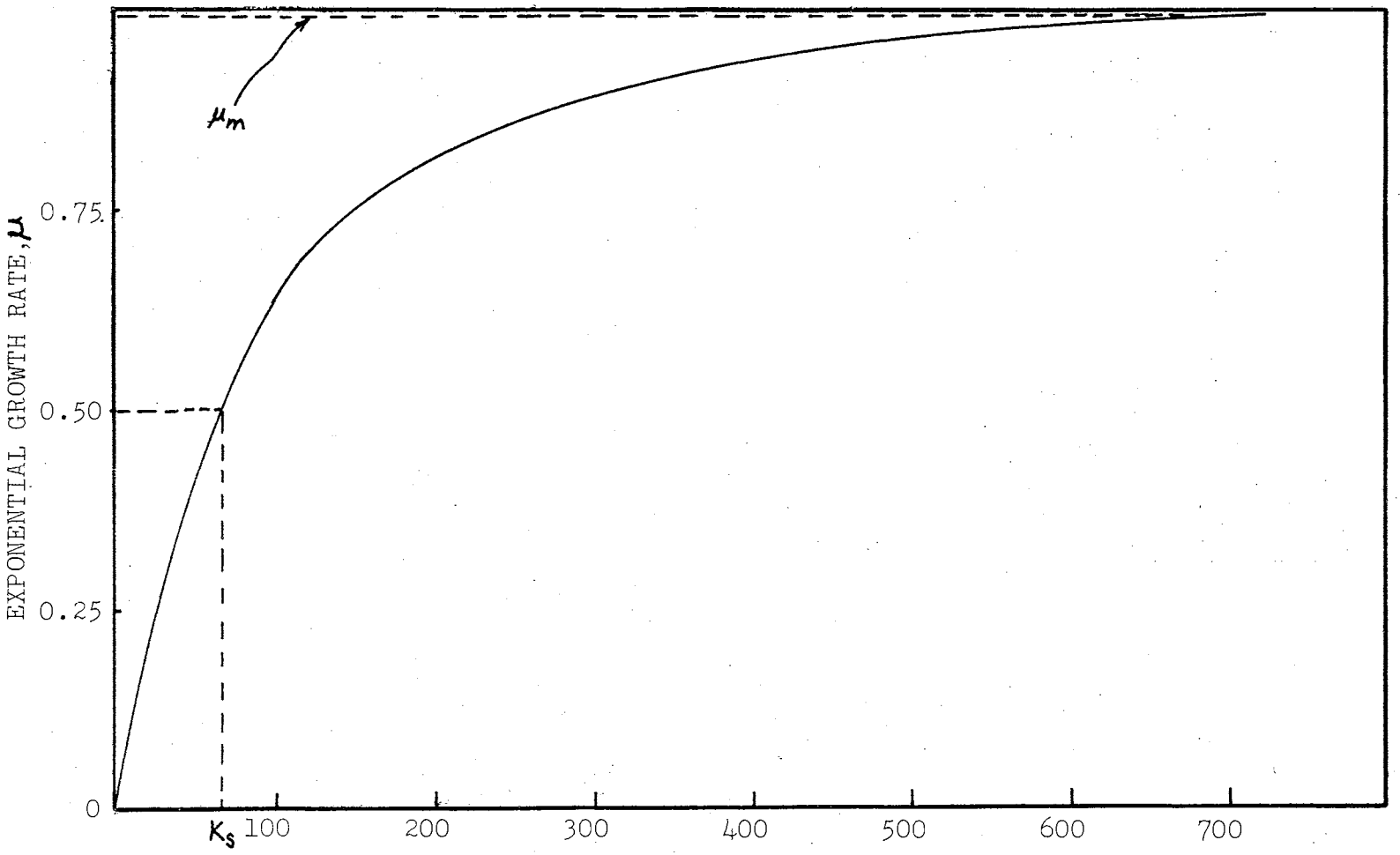

FIGURE 1 RELATION BETWEEN EXPONENTIAL GROWTH RATE (μ) AND CONCENTRATION OF LIMITING SUBSTRATE (s). AFTER HERBERT (35).

growth rate or:

$$\frac{dx}{dt} = \mu x \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (2)$$

where x is the concentration of organisms per unit volume. By combining equations (1) and (2) one obtains the following relationship between substrate concentration and the growth rate:

$$\frac{dx}{dt} = \mu_m x\left(\frac{s}{K_s + s}\right) \quad . \quad . \quad . \quad . \quad . \quad (3)$$

If Y is defined as $-\frac{dx}{ds}$ or weight of bacteria formed over the weight of substrate consumed, then equation (3) can be written as:

$$-\frac{ds}{dt} = \mu_m \frac{x}{Y}\left(\frac{s}{K_s + s}\right) \quad . \quad . \quad . \quad . \quad . \quad (4)$$

This is an expression for the rate of disappearance of the substrate.

Equations (3) and (4) hold true for batch and continuous flow systems since they express the rates of growth of the bacteria and disappearance of substrate from the medium.

For a more complete mathematical analysis of a continuous flow system without return of cells to the reactor, one must consider the loss of cells in the effluent. The cell balance equation would be:

Rate increase = rate of growth - rate of output

or

$$\frac{dx}{dt} = \mu_m x\left(\frac{s}{K_s + s}\right) - Dx \quad . \quad . \quad . \quad . \quad (5)$$

where D is the dilution rate or the reciprocal of the mean detention time in the reactor. When the system is in equilibrium, $\frac{dx}{dt}$ will be equal to zero and equation (5) reduces to:

$$D = \mu_m\left(\frac{s}{K_s + s}\right) \quad . \quad . \quad . \quad . \quad . \quad (6)$$

From equation (6) it is apparent that for all values less than the maximum growth rate, μ_m, the dilution rate, D, is equal to the growth rate, μ.

A substrate balance equation may be written as follows:

Decrease = input - output - consumption

or

$$-\frac{ds}{dt} = Ds_R - Ds - \mu_m\frac{x}{Y}\left(\frac{s}{K_s + s}\right) \quad . \quad . \quad (7)$$

In the steady state $\frac{dx}{dt}$ in equation (5) and $\frac{ds}{dt}$ in equation (7) are equated to zero, and equations (5) and (7) may be solved simultaneously to give:

$$s = K_s\left(\frac{D}{\mu_m - D}\right) \quad . \quad . \quad . \quad . \quad . \quad (8)$$

$$x = Y(s_R - s) \quad . \quad . \quad . \quad . \quad . \quad (9)$$

where s_R is substrate concentration in the incoming medium, s is the substrate concentration in the reactor and in the effluent (32). When μ_m, Y, and K_s are known, the steady state concentration of cells and substrate in a completely mixed reaction vessel may be predicted. These three constants must be measured in a batch system if x and s are to be predicted for a continuous flow system. If x and s are measured

for the continuous flow, then Y and K_s may be found from the steady state data.

The preceding relationships for batch and continuous culture of microorganisms are admittedly simplified ones. The questions raised by Contois (33) and Moser (34) are not considered because it is felt that they are beyond the scope of this investigation. Herbert (35) has obtained data which seems to fit the continuous flow formulae and Monod (30, 31) has presented data which fits the above batch formulae. On the strength of these findings, the author feels that these formulae should also suffice for the present study.

CHAPTER II

MATERIALS AND METHODS

Bacterial Culturing Equipment

The flasks used in the batch studies were of special design and are shown in Figure 2. The tubes, fitted to ground glass joints, were optically matched. The flasks were read by first inverting and then placing them into the spectrophotometer. Thus, no contamination was allowed to enter when the absorbancy of the systems was read. The flasks were placed on a shaking device which was equipped with a constant temperature bath. The temperature was maintained at 25°C. All absorbancies were read at 540 mμ on a Coleman model 6-D spectrophotometer using a 15 mm. (diameter) curvette.

The equipment used in the continuous flow studies consisted of a constant-head sterile medium reservoir, the Chemostat, an air filtration tube, an air-metering device, a 25°C. water bath, and a pump for pumping 25°C. water through the water jacket on the Chomostat (see Figure 3). The Chemostat was externally controlled by variation of the flow rate.

Analytical Techniques

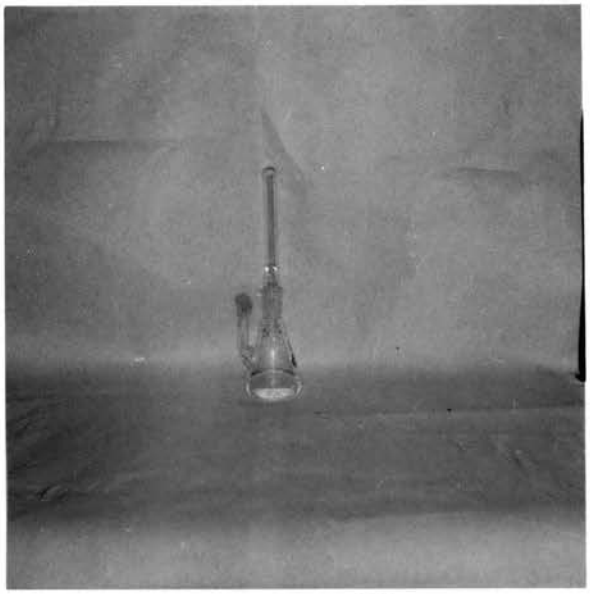

FIGURE 2 CULTURING FLASK USED IN DISCONTINUOUS STUDIES

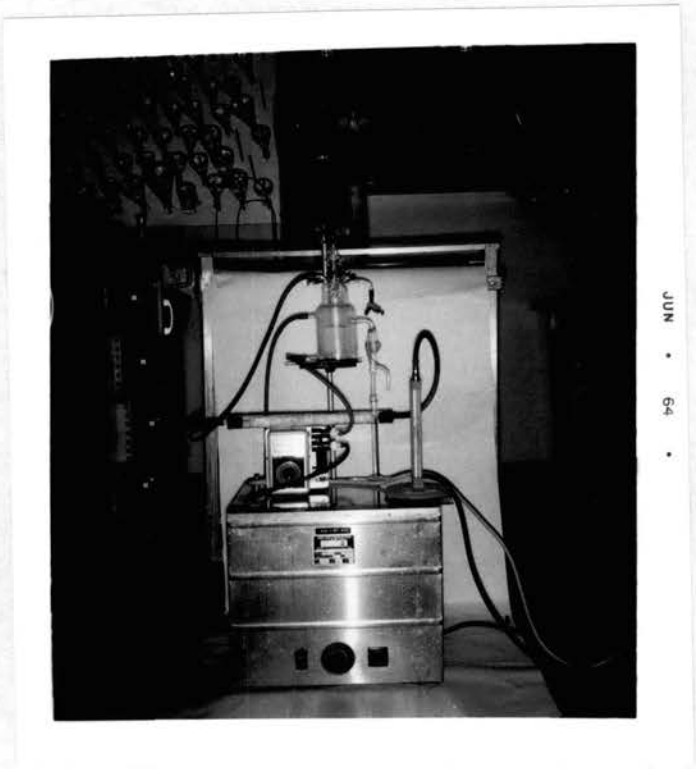

FIGURE 3 CONTINUOUS FLOW CULTURING APPARATUS

Determination of Viable Bacteria Population

Viable bacterial counts were obtained by the spot plate surface counting technique (22). After proper dilution with a physiological saline solution, an 0.08 ml. sample was placed on the surface of nutrient agar. By using approximately 0.02 ml. per spot, 4 spots per 1/2 plate were made so that a total of 8 spots were placed on each plate. In all cases no less than duplicate plates were made of each dilution that was counted. A statistical analysis of the surface plating technique has shown that this method is at least equivalent to the pour plate method (36).

Determination of Continuous Flow Effluent Residual Glucose

Residual glucose or the unused substrate in the effluent was measured by the Glucostat analysis technique. This test is an enzymatic color-forming reaction which is specific for glucose. The effluent was collected in a 10 ml. graduated cylinder which was packed in an ice bath. As the sample for the Glucostat analysis was collected, the flow rate of the Chemostat was measured as a check. The 10 ml. sample was filtered by using a membrane filter (0.45μpore size) and the filtrate immediately used in the glucose determination.

The instructions accompanying the Glucostat material were followed exactly for the running of the determination of glucose. This procedure is briefly described below.

The Glucostat reagents were prepared according to directions by dissolving the "chromogen" in 60 ml. of distilled water and by dissolving the enzyme in enough water to make the total volume 90 ml. Since the range of the test is most accurate with 0.05 mg./ml. to 0.25 mg./ml. of glucose, standards were prepared within this range. One milliter of the Chemostat effluent was used as the sample and duplicate samples prepared. The chromogen-enzyme mixture was added and, after ten minutes, one drop of 4 molar HCl was added to stop the reaction and fix the color. The tubes were read at a wave length of 400 mμ. A standard curve was plotted and the concentration of glucose determined from it.

Medium

The synthetic waste used for all growth studies consisted of the salts listed in Table I and glucose. For all continuous flow studies 500 mg./l. of glucose was used. For the batch studies the concentration of glucose was varied from 100 mg./l. to 1000 mg./l. The pH of the medium was always approximately 7.

Bacterial Cultures

Several cultures of organisms, which had been isolated from sewage or its receiving stream, were obtained from Dr. E. T. Gaudy of the O. S. U Department of Microbiology.

After growing these organisms on the glucose-salts medium, the decision to use the two organisms used in the study was made. Among the factors considered in their selection were, (1) ease of recognition of colonies on nutrient agar; (2) low affinity for adhering to the walls of the flasks; (3) growth rates which were different but not drastically different. One of the organisms was a strain of Serratia marcescens. The other organism gave small, smooth, flat, yellowish-white colonies on nutrient agar.

TABLE I

CONSTITUENTS OF SYNTHETIC WASTE

$(NH_4)_2SO_4$	500 mg./l.
$MgSO_4 \cdot 7H_2O$	100 mg./l.
$MnSO_4 \cdot H_2O$	10 mg./l.
$FeCl_3 \cdot 6H_2O$	0.50 mg./l.
$CaCl_2$	7.5 mg./l.
KH_2PO_4	526 mg./l.
K_2HPO_4	1070 mg./l.

Experimental Protocal

Batch Studies

A single inoculating loopful of organisms was removed from the surface of a nutrient agar culture and suspended in

60 ml. of medium containing 500 mg./l. of glucose. The culture was placed on the constant temperature shaking device and allowed to grow for 18 hours. One to five ml. of this culture was pipetted into the special flasks used for the batch studies. These flasks contained 60 ml. of glucose-salts medium. Duplicate flasks for each glucose concentration were used for all experiments. The inoculated special flasks were placed on the constant (25°C.) temperature shaker and measurements of the absorbancy were made at intervals of 30 minutes or 1 hour until the value became constant. Streak plates were made using nutrient agar and inocula from the flasks. This was done to check for purity and also to serve as a source of organisms for the next experiment.

Continuous Flow Studies

The entire arrangement of equipment was assembled and autoclaved with the exception of the water bath, pump, and air-flow meter. After cooling, the Chemostat was inoculated and the cultures allowed to grow with aeration (1000 c.c. per min.) for 18 hours. At the end of the 18-hour period a flow rate was set and samples of the effluent checked by means of reading their absorbancies. When three consecutive samples gave approximately the same reading, the unit was assumed to be in equilibrium. A sample of the effluent was collected and chilled, by means of an ice bath, and filtered (Millipore

HA, 0.45 μ). The filtrate was then analyzed for glucose using the Glucostat technique. After the effluent sample was collected, a sample was taken directly from the unit by means of a sterile 1-ml. pipette to assay for viable count. Dilutions were made using this sample, and an estimate of the viable bacterial population was obtained by using the surface plating technique. The flow rate was then changed to obtain the next higher dilution rate. Tests for purity of the cultures in the Chomostat were made by streaking a loopful of medium from the unit on nutrient agar.

CHAPTER III

RESULTS

Part 1. Effect of Substrate Concentration on Exponential Growth Rates of the Organisms in Discontinuous Systems.

In this series of experiments the substrate (glucose) concentration was varied (50-1000 mg./l.) to determine its effects on the exponential growth rates of the two organisms. At 50 mg./l. the growth curves for the organisms were not obtained because of the small increase in the number of cells. Growth curves were obtained for the two organisms at substrate concentrations of 100, 200, 300, 500, 750, and 1000 mg./l. The exponential growth rates from these data are shown in Tables II and III. Arithmetic plots of typical growth curves obtained at 100, 300, 500, and 1000 mg./l. substrate concentration are shown in Figures 4 and 5. Semi-logarithmic plots (see Figures 6 and 7) show the variation in the exponential growth rates with substrate concentration. The average value of the growth rate (see Tables II and III) at each substrate concentration studied is plotted in Figure 8. This plot of the data for S. marcescens shows that the maximum growth rate, μ_m is about 0.51. The K_s value is

TABLE II

GROWTH RATES FOR THE YELLOW ORGANISM
AT VARIOUS GLUCOSE CONCENTRATIONS

Glucose Concentration	Growth Rates (Log_e)	Average Growth Rate (Log_e)
1000	0.1546	0.1548
750	0.1428	0.1428
500	0.1423 0.1428 0.1456	0.1438
300	0.1001 0.1283 0.1801 0.1126	0.1300
200	0.1477 0.1470	0.1474
100	0.1111 0.1465	0.1288

TABLE III

GROWTH RATES FOR SERRATIA MARCESCENS
AT VARIOUS GLUCOSE CONCENTRATIONS

Glucose Concentration (mg./l.)	Growth Rates (Log_e)	Average Growth Rate (Log_e)
1000	0.4554	
	0.4717	
	0.4860	0.4893
	0.5124	
	0.5209	
900	0.5350	0.5092
	0.4835	
750	0.5350	
	0.5198	0.4945
	0.4577	
	0.4816	
500	0.4722	
	0.4986	
	0.5021	0.4738
	0.3820	
	0.5141	
300	0.5127	
	0.5081	0.4635
	0.4430	
	0.3798	
200	0.4943	
	0.4690	
	0.4970	0.4851
	0.4883	
	0.4768	
100	0.3740	
	0.4041	
	0.3712	0.3784
	0.3908	
	0.3521	

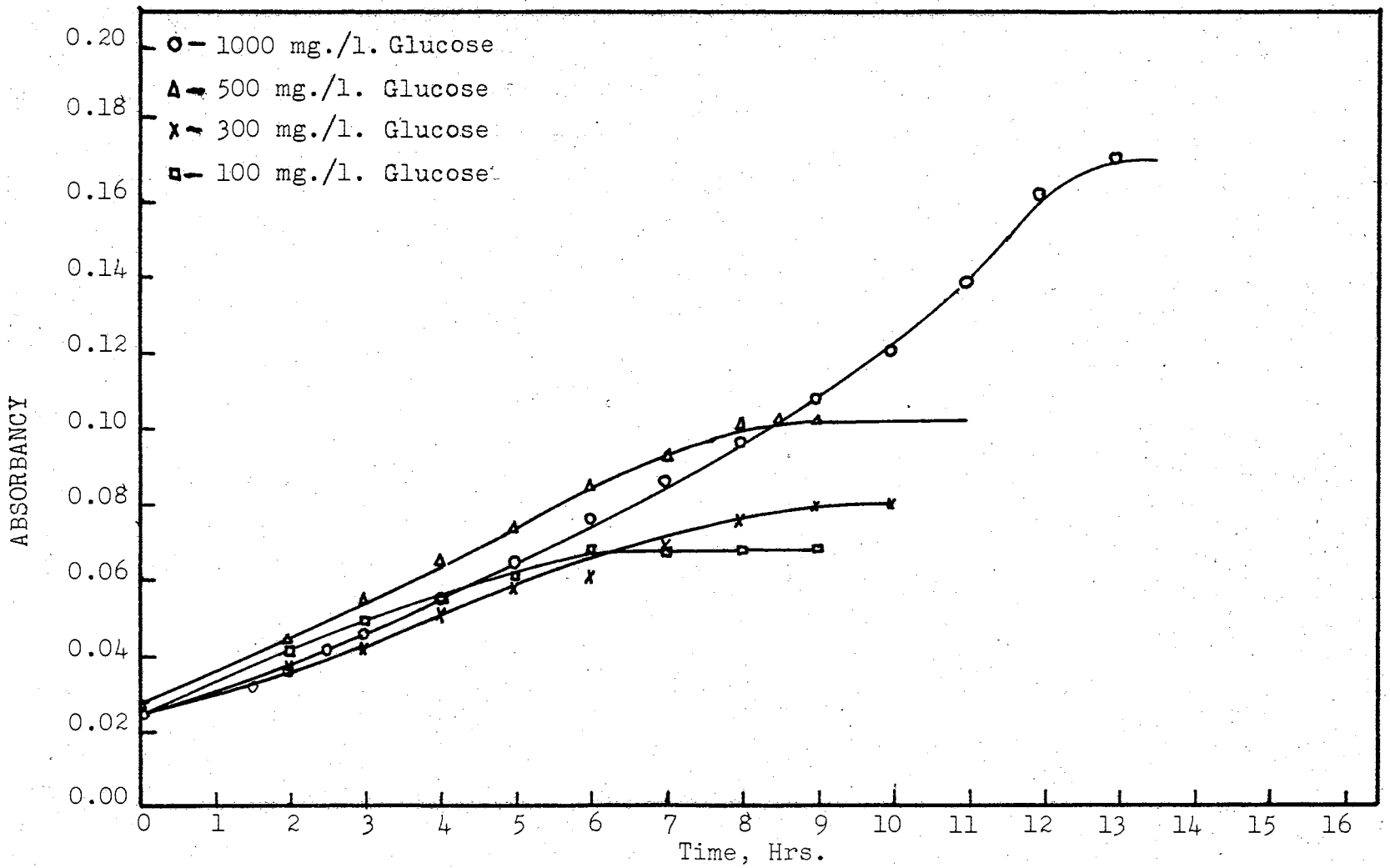

FIGURE 4 GROWTH CURVES FOR DISCONTINUOUS SYSTEMS USING THE YELLOW ORGANISM AT VARIOUS GLUCOSE CONCENTRATIONS

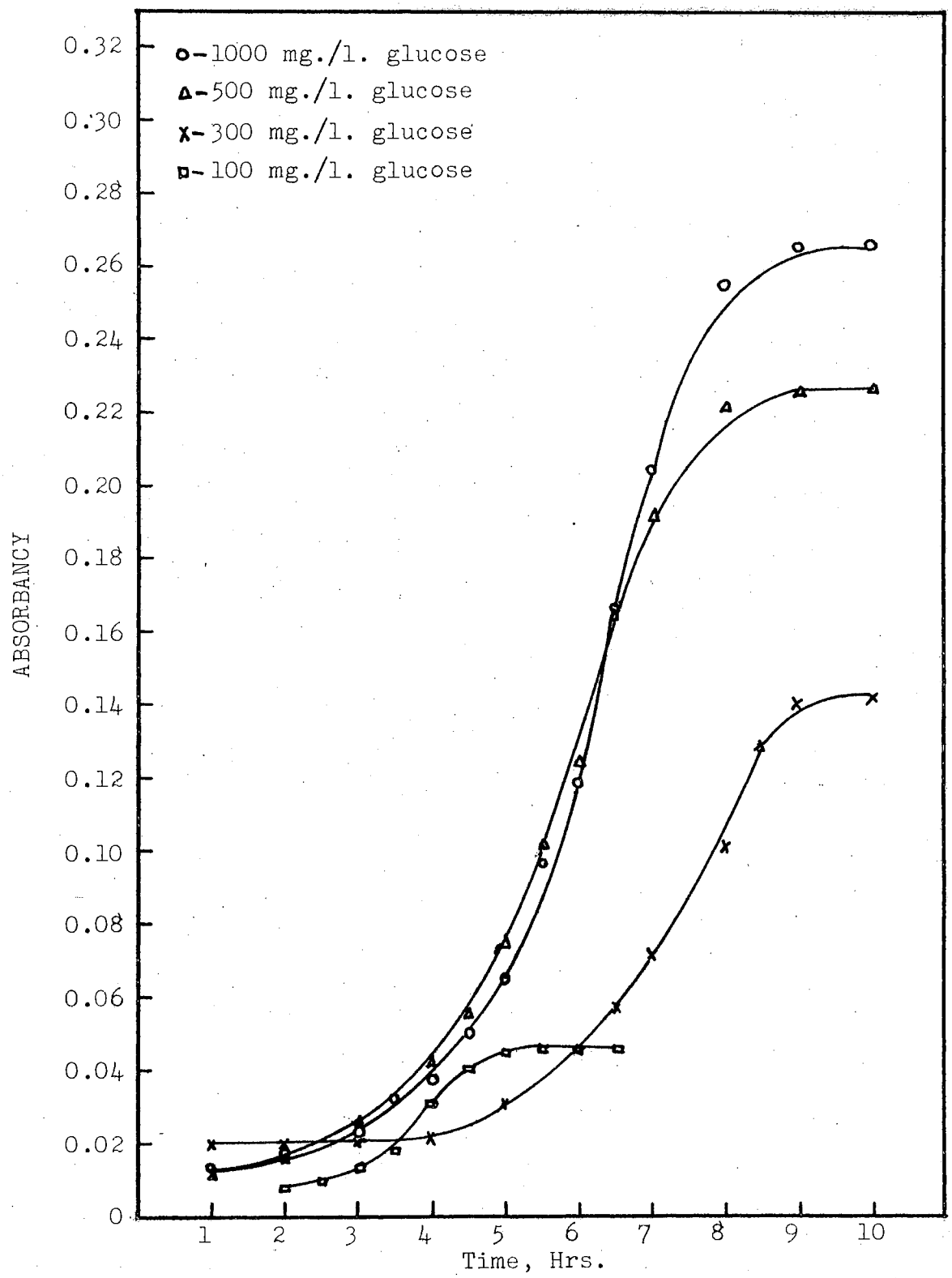

FIGURE 5 GROWTH CURVES FOR DISCONTINUOUS SYSTEMS USING S. MARCESCENS AT VARIOUS GLUCOSE CONCENTRATIONS

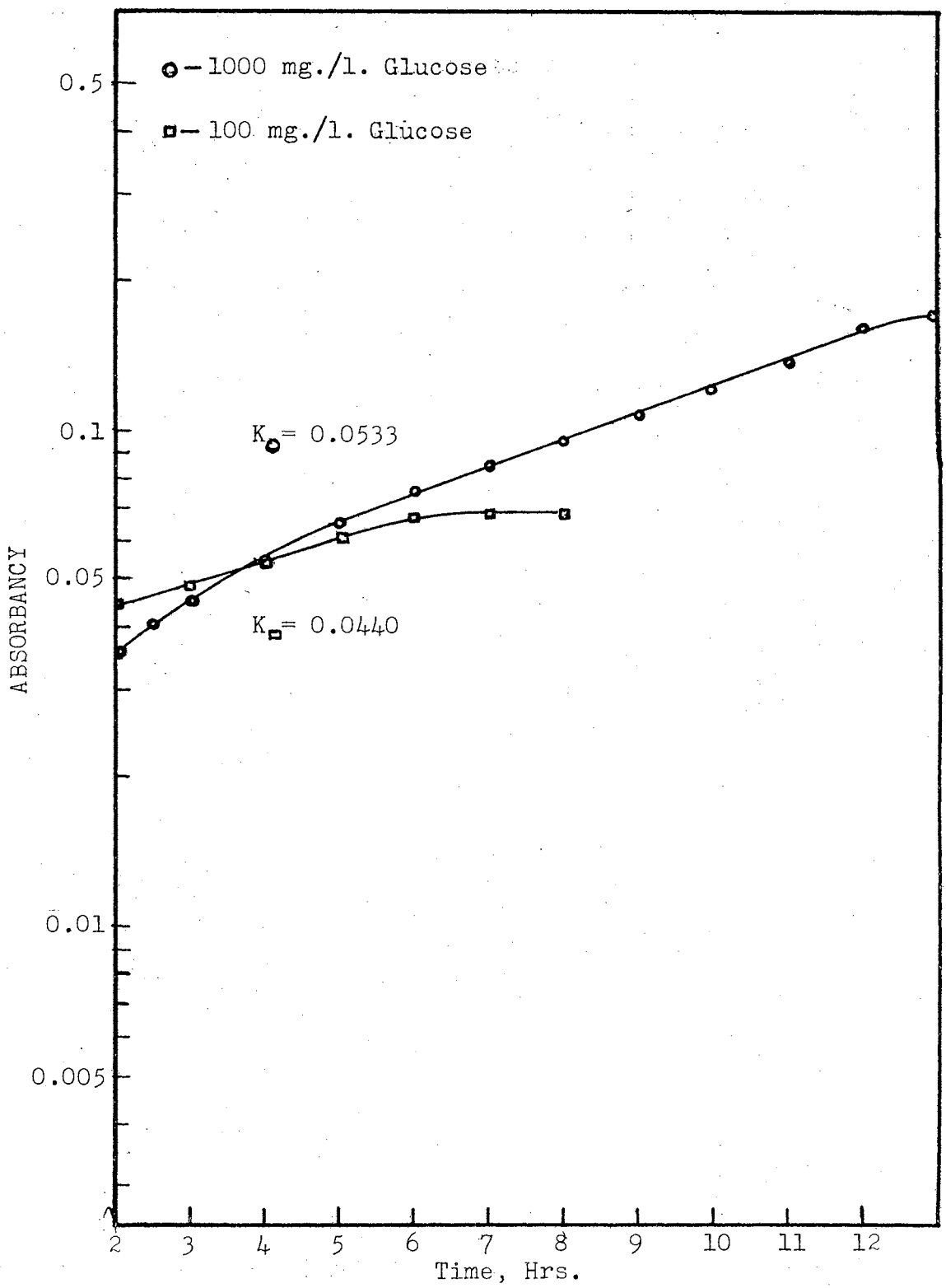

FIGURE 6 VARIATION OF LOG GROWTH RATE WITH SUBSTRATE CONCENTRATION FOR THE YELLOW ORGANISM

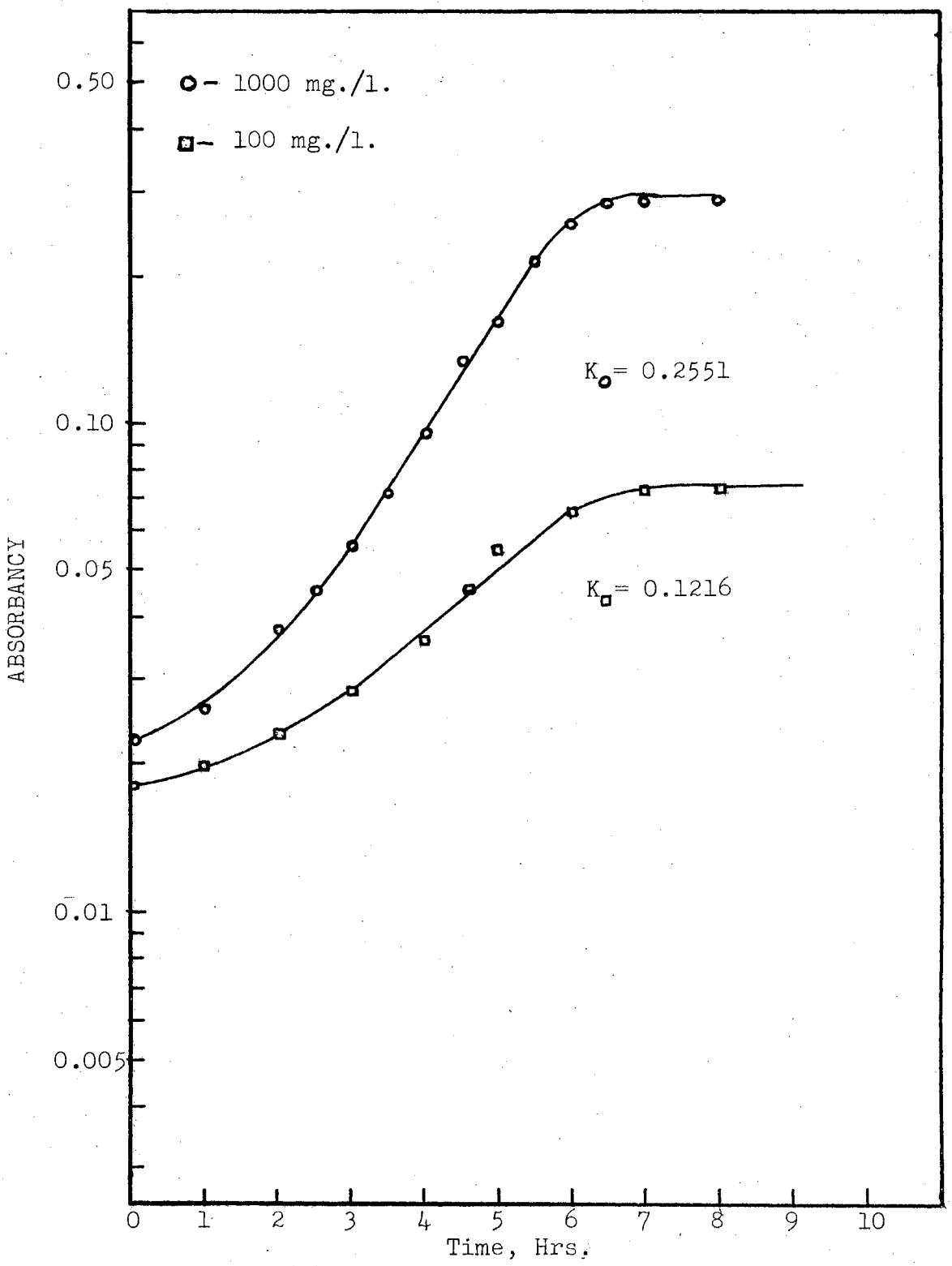

FIGURE 7 VARIATION OF LOG GROWTH RATE WITH GLUCOSE CONCENTRATION FOR SERRATIA MARCESCENS

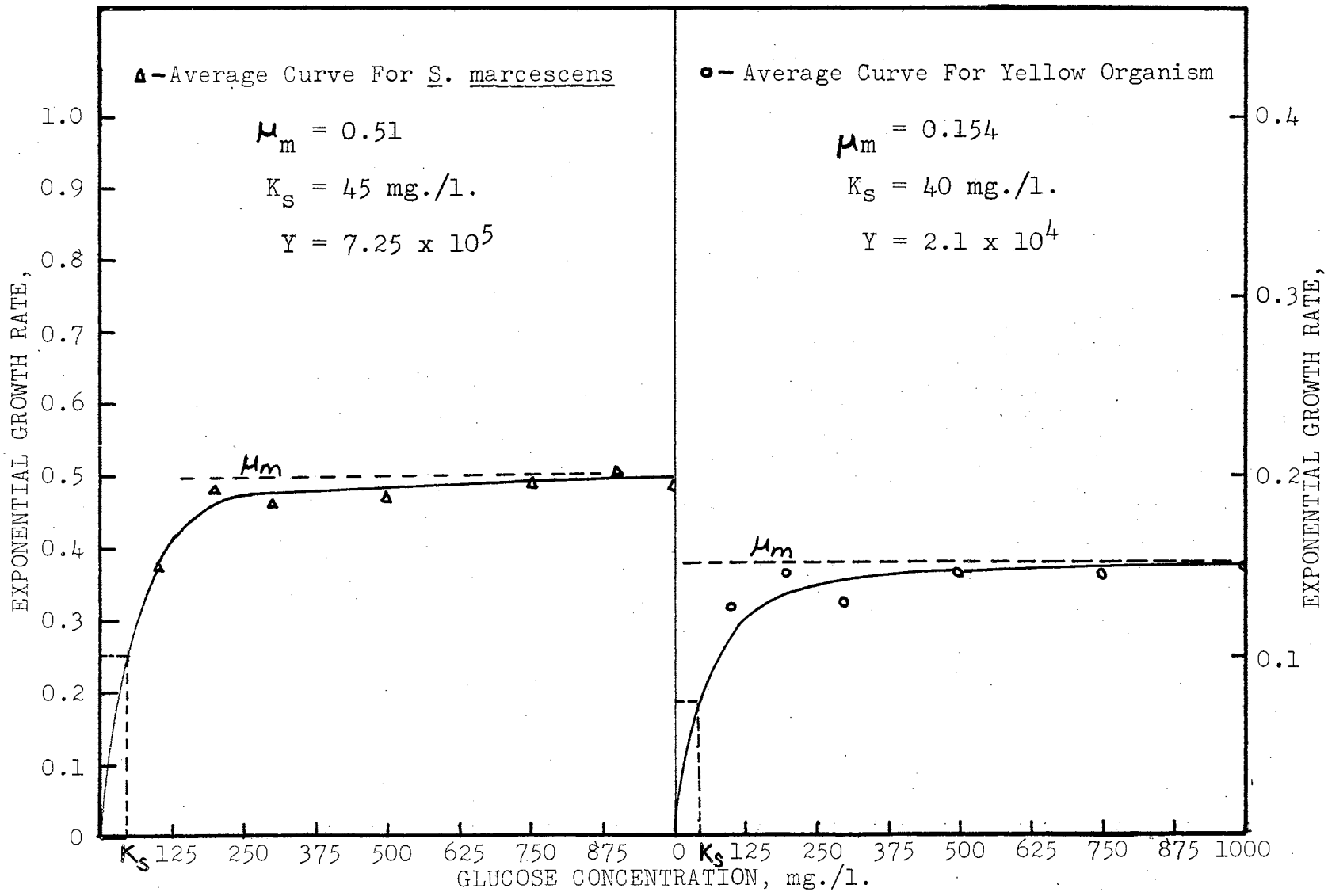

FIGURE 8 RELATIONSHIP BETWEEN GLUCOSE CONCENTRATION AND AVERAGE GROWTH RATE

about 45 mg./l. The plot of the data for the yellow organism shows μ_m to be about 0.154 and the K_s is the substrate concentration at one-half of the maximum growth rate value. Therefore, by reading the substrate concentration corresponding to 0.255 and 0.077, one would arrive at the stated K_s value. It should be noted that there are other ways of determining this important constant which may be employed as a check on the K_s value. An approximate check is presented in a later section of this report.

Part 2. Effects of Mixed Cultures on Growth Patterns of the Organisms in Discontinuous Systems.

The two organisms were cultured in exactly the same way as they were in the previous experiments except that mixtures of the two, as well as pure cultures of each organism, were examined. The results of two such experiments are given in Figures 9 and 10 and show the effects the bacteria exerted on each other while in mixed cultures. In both cases S. marcescens in the mixed culture reached approximately the same value as in pure culture. In mixed culture the yellow organism seemed to reach a peak value and then decline, whereas it did not in the pure culture. For the pure culture systems, an initial inoculum of 5 ml. of cell suspension was used, whereas for the mixed system, 2.5 ml. of each cell suspension was used. The question may arise as to whether or not the size

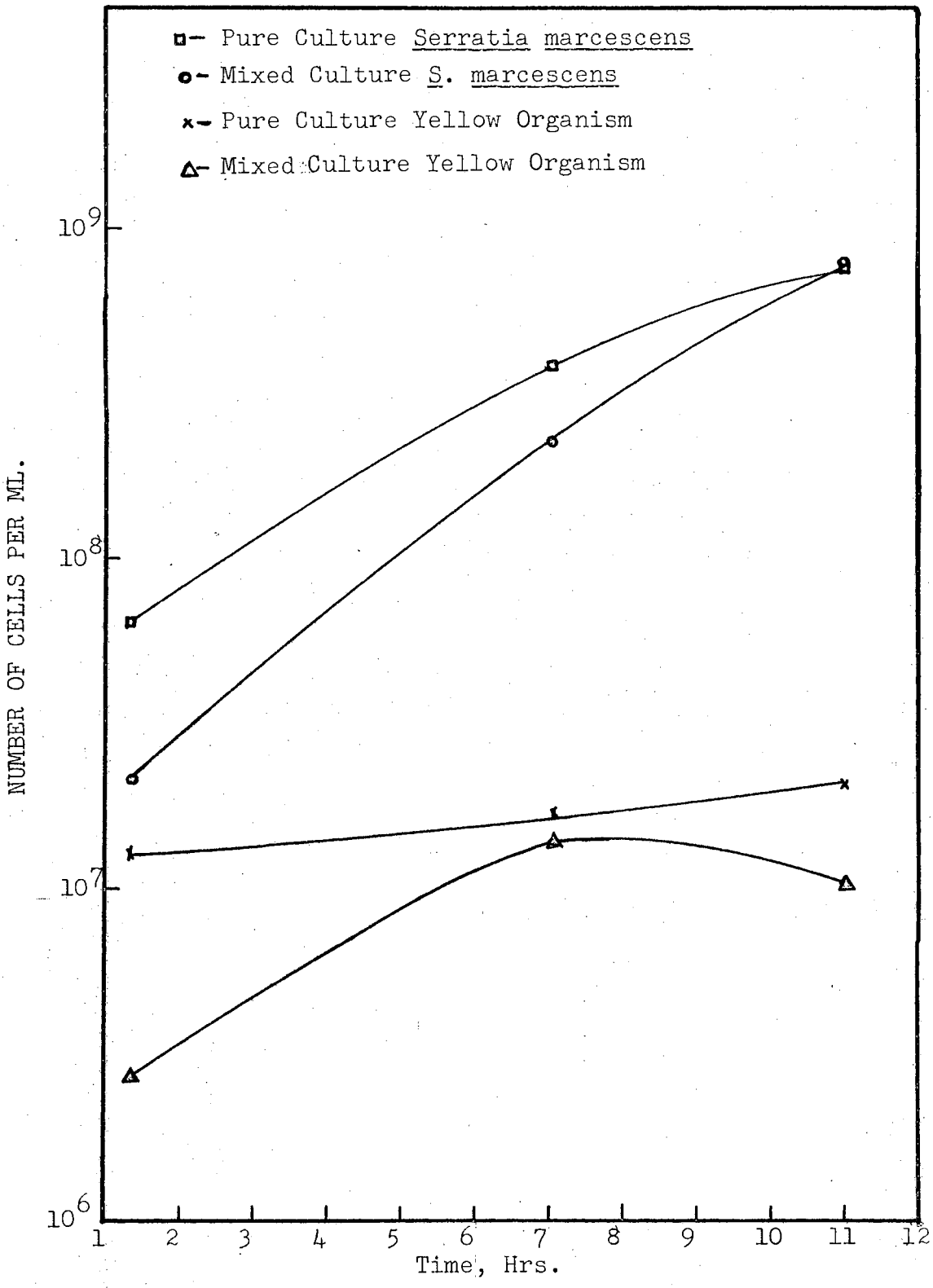

FIGURE 9 BEHAVIOR OF MIXED CULTURES OF THE YELLOW ORGANISM AND S. MARCESCENS

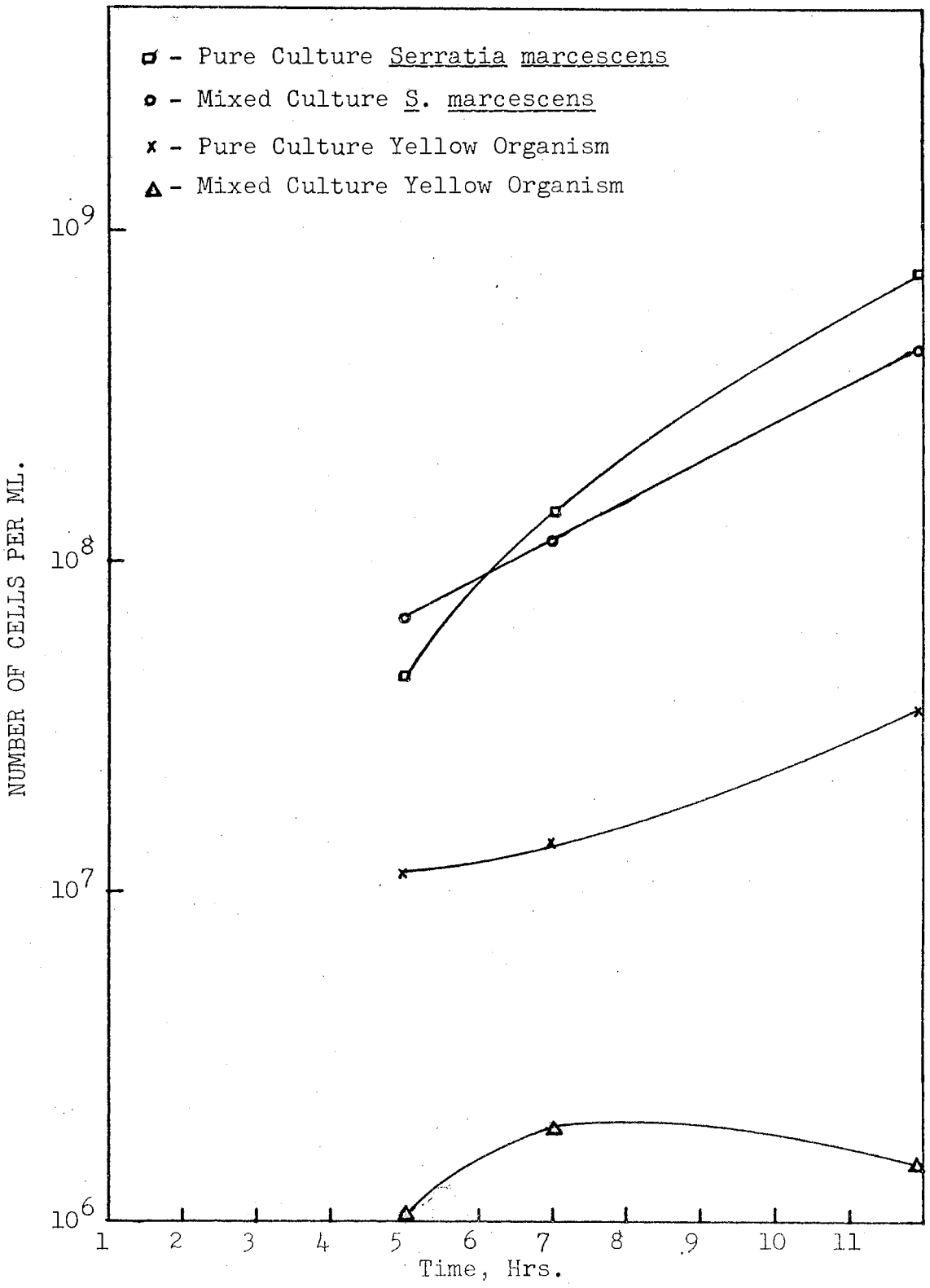

FIGURE 10 BEHAVIOR OF MIXED CULTURES OF THE YELLOW ORGANISM AND S. MARCESCENS

of the initial inoculum would affect the total growth of the system. The results of three experiments using different initial inocula of each organism are presented in Table IV.

TABLE IV

RELATIONSHIP BETWEEN INITIAL INOCULUM AND NUMBER OF CELLS PRODUCED ON 500 mg./l. SUBSTRATE

Organism	Initial Cell Concentration	Final Cell Concentration
S. marcescens	2.5×10^7	8.80×10^8
	8.0×10^7	9.35×10^8
	1.05×10^8	9.30×10^8
Yellow	3.8×10^6	3.60×10^7
	9.7×10^6	4.35×10^7
	1.8×10^7	4.05×10^7

It is seen that, in the range examined, the size of the initial count did not materially affect the final cell concentration.

Part 3. Determination of Kinetic Constants and Growth Patterns of the Organisms in Continuous Flow Systems.

As has already been noted, a plot of the cell concentration and substrate remaining against the dilution ratio may by theoretically obtained by the use of equations (8) and (9). The values of μ_m, K_s, and Y may be determined in discontinuous

systems and used to calculate s and x for a continuous (steady state) system. If the initial and final concentration of cells and substrate are known, Y can also be determined from batch data. Table V gives the results of a typical experiment for each organism. If a continuous flow system is used, then Y may also be determined by knowing the input and effluent substrate concentrations and the number of cells present in the effluent. Table VI shows cell yield obtained from four experiments on S. marcescens and one on the yellow organism using steady state data. Knowing μ_m, K_s, and Y, the cell concentration and effluent substrate concentration for various dilution rates can be computed. Using values of K_s and μ_m obtained from Figure 8 and average Y values from Table VI, the value of s and x were computed for various dilution rates (see Table VII) and are plotted for both organisms in Figures 11 and 12. In addition to the theoretical (computed) values, the actual experimental values of s and x obtained at various dilution rates are shown in Figures 11 and 12. The experimental data are also given in Tables VIII and IX.

With the values of μ_m (from Figure 8), s, and D (from steady state data), K_s may be calculated. These calculated values of K_s are approximate checks on the values taken from Figure 8 (see Table X).

Part 4. Effects of Mixed Cultures on Growth Patterns of the Organisms in Continuous Flow Systems.

TABLE V

CELL YIELD OBTAINED IN DISCONTINUOUS SYSTEMS

Organism	s^* (mg./l.)	c_1 (Cells/ml.)	s_2 (mg./l.)	c_2 (Cells/ml.)	Y (Cells/mg.)
S. marcescens	500	3.62×10^7	6	3.94×10^8	7.25×10^5
Yellow	530	6.37×10^6	470	7.62×10^6	2.1×10^4

* Subscripts 1 and 2 indicate initial and final cell and substrate concentration obtained during the log phase of growth.

TABLE VI

CELL YIELD OBTAINED IN CONTINUOUS SYSTEMS

Organism	s_R (mg./l.)	s (mg./l.)	x (Cells/ml.)	Y (Cells/mg.)
S. marcescens	500	39	1.28×10^8	2.75×10^5
	500	55	9.6×10^7	2.18×10^5
	500	41	1.75×10^8	3.8×10^5
	500	24	8.55×10^7	1.8×10^5
Yellow	500	5	3.27×10^7	6.6×10^4

TABLE VII

THEORETICAL VALUES OF KINETIC CONSTANTS AND VARIABLES

Organism	Dilution Rate	m Growth Rate Constant (Batch)	K_s (Batch)	Yield (Cont. Flow)	s Effluent Glucose Conc. (mg./l.)	s_R-s Initial Minus Final Glucose Conc. (mg./l.)	x No. of Cells/ml.
S. marcescens	0.1	0.51	45	2.29×10^5	7.5	493	1.130×10^8
	0.2	0.51	45	2.29×10^5	13.0	487	1.110×10^8
	0.3	0.51	45	2.29×10^5	17.0	483	1.105
	0.4	0.51	45	2.29×10^5	20.0	480	1.100
	0.5	0.51	45	2.29×10^5	22.5	478	1.095
	0.6	0.51	45	2.20×10^5	24.6	476	1.090
	0.7	0.51	45	2.29×10^5	26.0	474	1.085
	0.76	0.51	45	2.29×10^5	27.2	473	1.083
Yellow	0.05	0.153	40	2.5×10^4	9.9	490	1.25×10^7
	0.10	0.153	40	2.5×10^4	15.8	484	1.21×10^7
	0.15	0.153	40	2.5×10^4	19.8	480	1.20×10^7
	0.20	0.153	40	2.5×10^4	22.6	477	1.19×10^7
	0.22	0.153	40	2.5×10^4	23.8	476	1.19×10^7

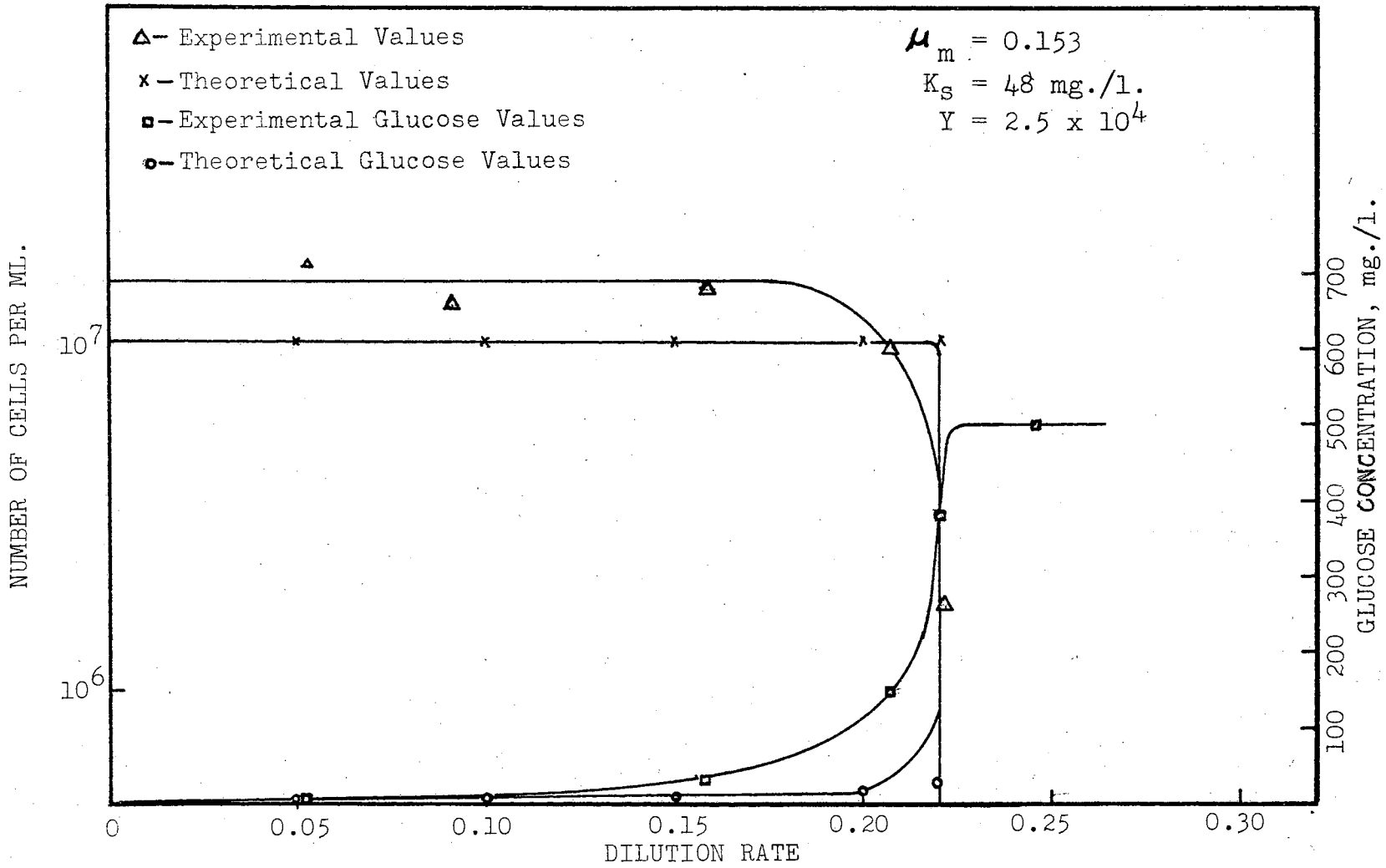

FIGURE 11 COMPUTED AND EXPERIMENTAL DILUTE-OUT CURVES FOR THE YELLOW ORGANISM IN A CONTINUOUS FLOW SYSTEM

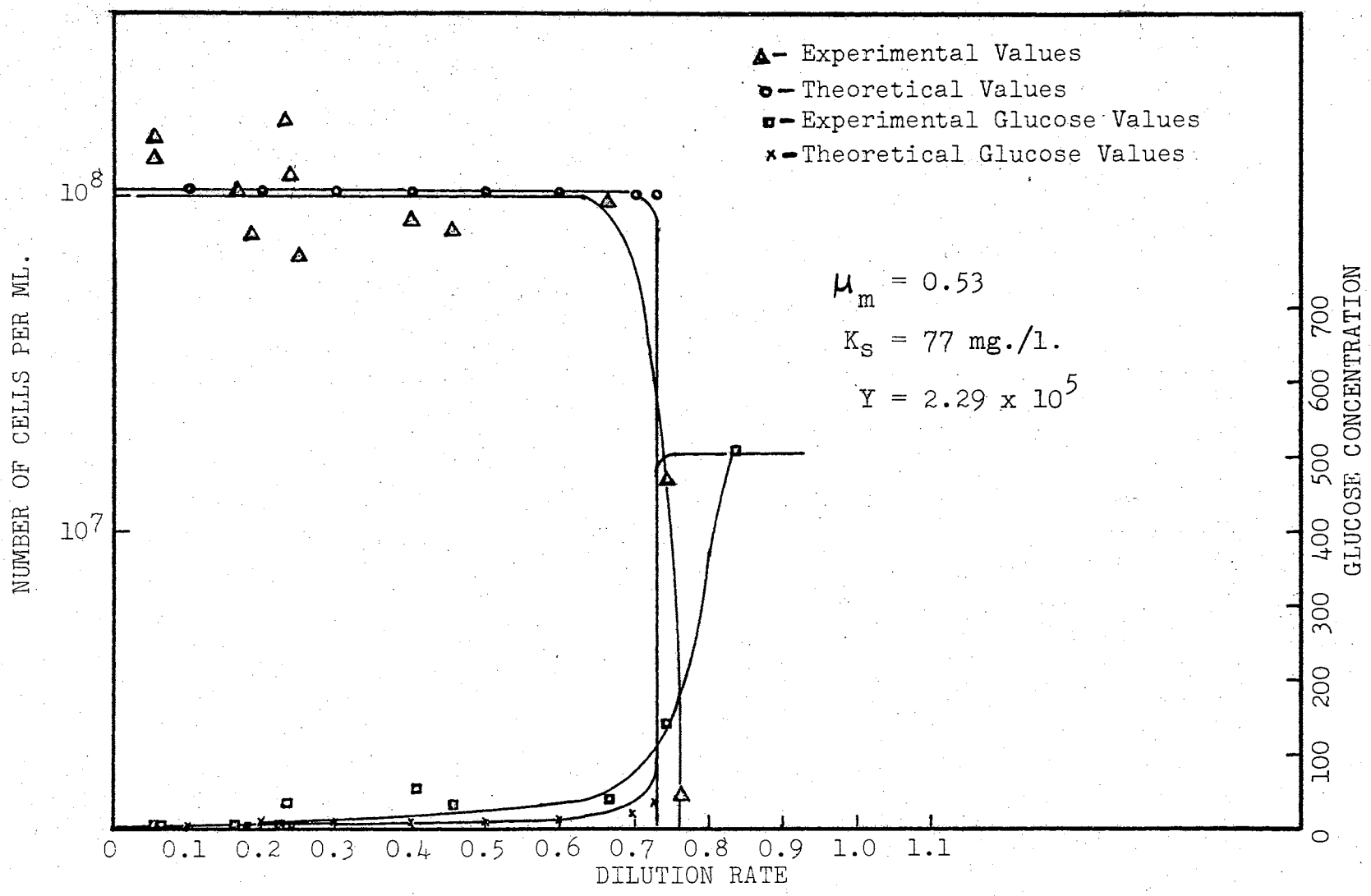

FIGURE 12 THEORETICAL AND EXPERIMENTAL DILUTE-OUT CURVES FOR S. MARCESCENS IN A CONTINUOUS FLOW SYSTEM

TABLE VIII

DILUTION RATE, EFFLUENT GLUCOSE CONCENTRATION

YELLOW ORGANISM						
Flow Rate (ml./hr.)	Dilution Rate	s* (mg./l.)	N+	Colonies Per 1/2 Plate		Organisms Per Ml.
6.4	0.0512	0+	5	22	25	3.27×10^7
				29	29	
11.2	0.0918	0+	4	170	144	2.3×10^7
				139	160	
19.3	0.158	33	5	27	17	
			4	240	260	2.75×10^7
				245	255	
25.2	0.206	144	4	94	99	1.15×10^7
				90	85	
26.0	0.221	382	4	31	19	3.35×10^6
				37	29	
30.0	0.246	503	3	0	0	$>10^3$
				0	1	

* s is the effluent glucose concentration.

\+ N is equal to the dilution factor (10^{-N}).

TABLE IX

DILUTION RATE, EFFLUENT GLUCOSE CONCENTRATION, NO. OF ORGANISMS DATA

SERRATIA MARCESCENS						
Flow Rate (ml./hr.)	Dilution Rate	s* (mg./l.)	N+	Colonies Per 1/2 Plate		Organisms Per Ml.
5.8	0.054	0+	6	26 17	24 19	2.69×10^8
6.2	0.057	0+	5	154 162	160 185	2.03×10^8
18.0	0.167	0+	5	102 108	108 110	1.35×10^8
20.0	0.185	0+	5	72 81	73 80	9.0×10^7
24.0	0.231	0+	6	29 19	23 31	3.12×10^8
25.8	0.239	41	5	136 150	133 153	1.75×10^8
27.1	0.251	24	5	81 65	67 61	8.55×10^7
43.0	0.398	55	5	76 79	84 73	9.6×10^7
49.2	0.455	39	5	72 73	75 71	9.12×10^7
72.0	0.667	41	5	82 80	79 79	1.0×10^8
80.0	0.741	143	5	36 17	20 18	2.5×10^7
82.0	0.760	--	4	21 27	27 21	3.0×10^6
90.0	0.834	510	4	0	0	$>10^5$

* s is the glucose concentration in the effluent.

\+ N is equal to the dilution factor (10^{-N}).

The same apparatus and culturing techniques were employed in this series of experiments as in the continuous flow pure culture experiments except that a mixture of both organisms was used. The units were seeded with approximately equal amounts of the yellow organism and S. marcescens. Table XI shows the steady state cell counts obtained with mixed cultures. Figure 13 shows a comparison of this mixed culture data and the data obtained when each organism was grown in pure culture. It is seen that the yellow organism was diluted out of the mixed culture system at a dilution rate of about 0.15. The maximum concentration of the yellow organism in mixed culture was about 4.0×10^6 cells/ml., while in pure culture, it was about 2.5×10^7 cells/ml. The S. marcescens maintained approximately the same concentration of cells in mixed culture as in pure culture (approximately 2.3×10^8 cells/ml.).

TABLE X

EXPERIMENTAL VALUES OF S AND K_s AT VARIOUS DILUTION RATES

Organism	Dilution Rate	μ_m	K_s (mg./l.)	s (mg./l.)
S. marcescens	0.251	0.53	74	24
	0.398	0.53	74	24
	0.455	0.53	85	39
Yellow	0.052	0.153	27	7
	0.158	0.153	69	35

TABLE XI

NUMBER OF EACH ORGANISM IN MIXED CULTURE

Flow Rate (ml./hr.)	Dilution Rate	Number of S. marcescens Per Ml.	Number of Yellow Organisms Per Ml.
5.5	0.05	4.0×10^8	4.06×10^6
9.0	0.074	2.2×10^8	3.93×10^6
13.5	0.125	2.28×10^8	3.5×10^6
18.0	0.147	3.80×10^8	1.72×10^6
20.0	0.164	2.12×10^8	$>10^6$
30.0	0.246	1.01×10^8	$>10^5$

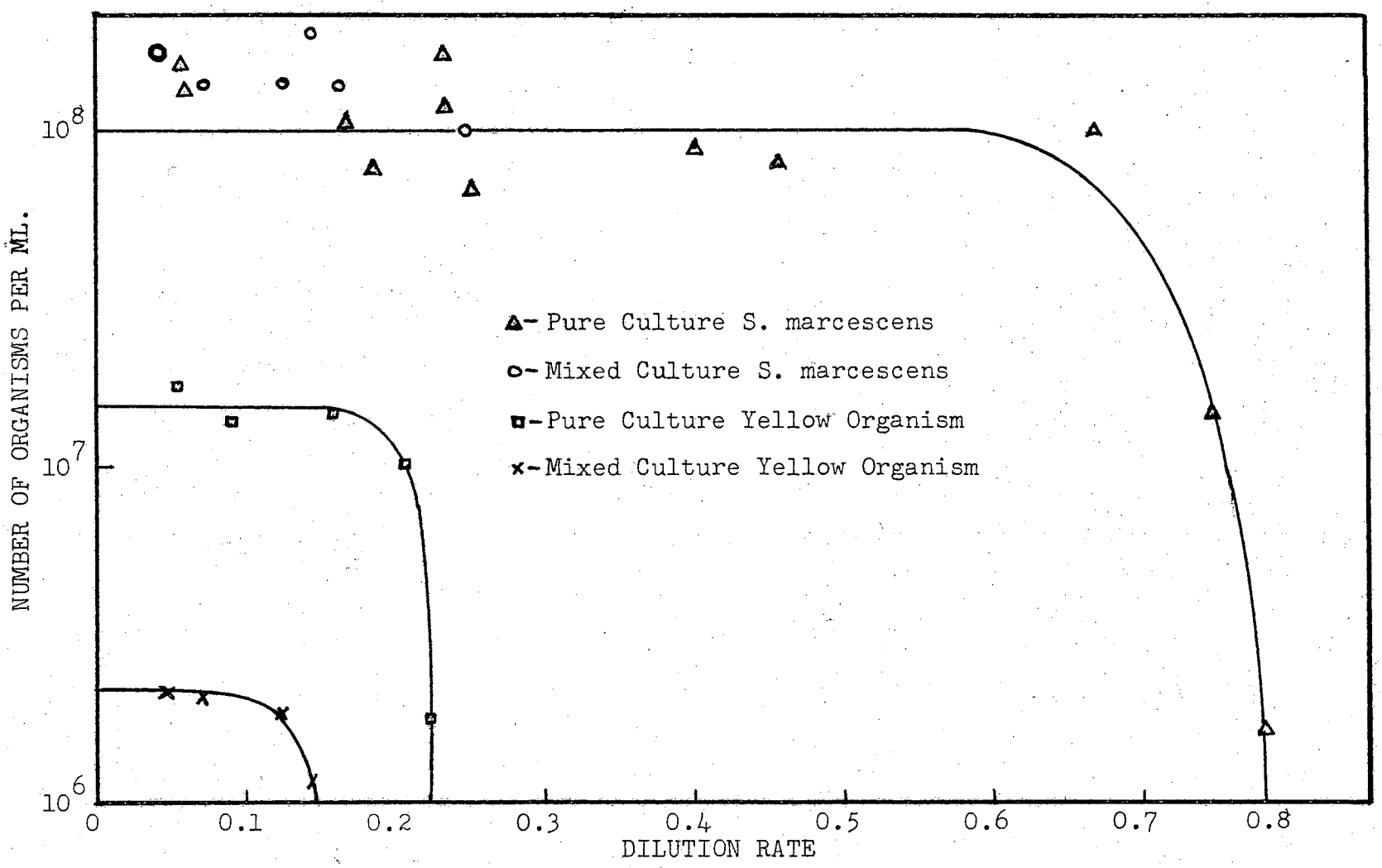

FIGURE 13 NUMBER OF ORGANISMS IN PURE AND MIXED CULTURES AT VARIOUS DILUTION RATES

CHAPTER IV

DISCUSSION

The results of the study on the effect of the substrate concentration on the exponential growth rate show that the growth rate is dependent on the limiting substrate concentration up to a critical value. The yellow organism exhibited a growth rate at 100 mg./l. which is about 17 per cent less than its growth rate at 1000 mg./l. The growth rate at 100 mg./l. for S. marcescens was about 23 per cent less than its growth rate at 1000 mg./l. Several attempts were made to obtain growth curves of the organisms at 50 mg./l., but reliable results could not be obtained. The number of cells produced could not be measured accurately enough to make plots of the growth rates. The question may arise as to why approximately the same initial inoculum was used for experiments below 100 mg./l. as above 100 mg./l. It would seem that the slope of a semi-log plot of cell concentration versus time could be more accurately determined. This is true, but the data of Fujimoto (37) indicates that when a very small inoculum (about 10^3 or 10^4 cells/ml. of Escherichia coli) was used, the growth curves had a steeper slope in the log growth phase

than when a larger initial inoculum (10^7 cells/ml.) was used. Therefore, in the interest of making valid comparison, it seemed advisable that the initial inoculum be in the same range as the other experiments. When this range was used, an insufficient number of cells was produced to assure an accurate estimate of the change in population. The growth rate at 100 mg./l. for S. marcescens is 0.3784, while it is 0.4851 at 200 mg./l. and 0.4893 at 1000 mg./l. (see Figure 8). Therefore, the value at which there was no appreciable increase in lies between 100 and 200 mg./l. For the yellow organism, this substrate concentration is not easily apparent but is probably between 100 and 500 mg./l.

When the two organisms were mixed in discontinuous systems, the S. marcescens grew as well as if there were no other organism present. The yellow organism reached a maximum value below the pure culture value and then declined (see Figures 8 and 9). Since the yellow organism grew, it had to utilize some energy source. If this source was not the glucose but something else (perhaps an end product or intermediate metabolite of the S. marcescens), then one might expect that growth would have continued to increase with time. If, on the other hand, the organism did use the glucose, the observed result would be expected, i.e., growth would continue until there was no longer any exogenous energy source and then go through

a stationery phase in which storage products may be used, and, finally, the viable population would decline. The result indicates that the presence of S. marcescens adversely affected the attainment of maximum population of the yellow organism. It is not possible from these data to determine whether this adverse effect was due simply to substrate depletion by S. marcescens or to an antagonistic mechanism. However, one experiment that was designed to show possible antagonism indicated that S. marcescens did exert an antagonistic effect. The experiment was carried out by first making a pour plate of the yellow organism and then placing four 0.02 ml. drops of a S. marcescens aqueous cell suspension on the pour plate. Although the four drops of S. marcescens flowed together on the wet agar, a definite zone of greatly reduced growth of the yellow organism could be seen around the periphery of the S. marcescens growth. This reduced growth is believed to be caused by some antagonistic agent liberated into the agar by the S. marcescens.

The reduced growth of the yellow organism in the mixed discontinuous system with the S. marcescens shows a similar result as the pour plate experiment.

Although the initial viable population was different for the pure culture and mixed culture, it is felt that the systems can be directly compared since it may be surmised from

Table IV that the size of the initial inoculum did not affect the total production of cells. This observation is also substantiated by the data of Fujimoto (37).

The results of the experiments with mixed cultures in the continuous flow systems also show a definite predominance of the S. marcescens over the yellow organism. One might expect this, as its maximum growth rate has a greater value. The two organisms do not grow independently of one another, however. The critical dilution rate of the yellow organism was found to be 0.221 in pure culture, while in mixed culture it appears to be about 0.15. The cell concentration of the yellow organism is about 2.6×10^7 cells/ml. in pure culture, while it is only about 3.8×10^6 cells/ml. in mixed culture. The S. marcescens maintains an almost constant concentration in mixed and pure cultures (see Figure 13). The mean generation time of the yellow organism is increased in mixed culture if one takes the apparent critical dilution rate and back calculates the mean generation time. The level of the cell concentration of the yellow organism was about 10 times as great in pure culture as in mixed culture. The S. marcescens was obviously more successful in the competition, since its cell concentration level remained approximately the same in mixed as in pure culture. The exact cause of this apparent antagonistic effect of S. marcescens on the yellow organism was not determined.

However, examination of the spot plates of the mixed cultures showed that the yellow colonies would grow less rapidly in the direction of contact with S. marcescens. Indeed, it was observations such as this which indicated the advisability of making the experiment to gain some insight into the antagonism which was previously described.

As shown in Figures 11 and 12, the plots of the theoretical curves for the continuous flow systems are in close agreement with the plots of the experimental values obtained. The dilution rate at which the cells would be washed out of the Chemostat was predicted to be 0.73 from the discontinuous flow data for S. marcescens in pure culture. The actual value was 0.76. The predicted wash-out value was computed in the following manner. Since the value of μ_m (obtained from the discontinuous flow studies) was 0.51, the mean generation time was $\log_e 2/0.51$ or 1.37 hrs. The critical value for the dilution rate would then be 1/1.37 or 0.73. The predicted critical dilution rate for the yellow organism was 0.222, while the actual value found was 0.221. The close agreement of these values is good evidence for the correctness of the μ_m and K_s values obtained from the discontinuous flow studies.

The yield values used in the computation of the theoretical values of cell concentration (see Table VII and Figures 11 and 12) were from the continuous flow systems. It was felt

that the value of the yield constant should be taken from the continuous flow data because a greater amount of steady state data was available. According to the Monod concept (29, 30, 31), there should be no difference in the yield values of the two systems, since the yield is assumed to be constant when the same substrate is fed to the organism. From the data herein reported, it cannot be said that the yield in the batch system was the same as that obtained from the steady state data.

The values calculated from the continuous flow data for K_s are, in general, higher than the values taken from the discontinuous data. The value for S. marcescens is about 77 mg./l. and for the yellow organism about 48 mg./l. as compared to 45 mg./l. and 40 mg./l. from the discontinuous data. The calculations for K_s in continuous flow are based on a calculated μ_m from the continuous flow data and the concentrations of the effluent glucose, (s).

One method of calculating μ_m for S. marcescens from the continuous flow data would be as follows: Take the maximum dilution rate from the experimental dilute-out curves (see Figures 10 and 11), which would be 0.76. Equate this value to the reciprocal of the mean generation time, 1/t. Solve for t and obtain 1.31 hrs. This value is now equal to $\log_e 2/\mu_m$ which yields μ_m to be 0.53.

The difference in the values of K_s are not very great when the difficulty with which they were measured in the batch systems is considered.

The author feels that a brief discussion of some of the difficulties encountered in research of this type and some mention of the preliminary studies which preceded the work may be of value to those wishing to carry this line of investigation forward.

Nine experiments on Pseudomonas fluorescens were carried out before it was decided that the adherence of the cells to the walls of the flasks was so excessive that no reliable results could be obtained. In addition, a few of the results from the 18 experiments conducted using the organisms herein reported had to be discarded because of equipment failure. One experiment was discarded due to contamination of the cultures. Contamination was easily detected because of the distinct morphological characteristics of the colonies. Also, streak plates were made at the end of each experiment to check for the purity of the culture before the results of that experiment were accepted. The culturing medium had to be autoclaved each time in exactly the same manner since the losses due to evaporation of the fluid caused a variation of the glucose concentration in the culturing flasks used in the discontinuous flow systems. Since an initial inoculum of 5 ml. of

cell suspension was used in most of the experiments in discontinuous systems, only very little glucose could remain in the seed culture at the time the cell suspension was removed for inoculating. Therefore, it was found necessary to incubate the seeding culture longer than necessary for the culture to be in its most active stage. As a result, somewhat extended lag periods were observed. If a heterogeneous population had been used, the seed culture could have been harvested and washed free of glucose without incurring an exceedingly laborious procedure. Naturally, a heterogeneous culture would have been of no value to this study and is only mentioned to point out the relative difficulty involved when using small amounts of pure culture seed. Since absolutely no contamination could be tolerated during the experiments, large numbers of sterile pipettes, dilution bottles, and petri dishes were needed, especially on the mixed culture experiments.

The main problems encountered in the continuous flow experiments were: (1) maintaining a constant flow of medium; (2) side-wall growth; (3) maintaining the system free from contamination; and (4) maintaining a constant supply of sterile medium. Solution metering pumps (in the flow rate range required) failed completely. Under extended continuous operation, they would either stop pumping or develop leaks. The constant-head-siphoning device was very sensitive to large

changes in the room temperature caused by other experiments in progress. Frequent checks of the flow rate were required.

The Chemostat had to be carefully cleaned to retard side-wall growth. While the yellow organism never exhibited any side-wall growth except in the outflow tube, the S. marcescens did exhibit side-wall growth after several days of operation. The equipment would then have to be washed and the apparatus resterilized and reinoculated. It may be interesting to note that neither organism accumulated above the surface of the medium on the walls of the flask (because of the splashing and bubbling produced by aeration). However, when growth in this region was observed, it was a certain indication of contamination. Also, the side-wall growth in the S. marcescens unit was always red unless contamination was present. Another observation that may be of interest was the accumulation of red pigment on the sides of the plastic beaker used to collect the effluent from the S. marcescens unit. When the two organisms were mixed, little or no pigment collected on the sides of the beaker even though it has been shown that there were approximately the same number of S. marcescens in the effluent at all times the unit was in equilibrium. A possible cause could have been "sorption" of the red pigment to the yellow organisms, which kept the pigments from collecting on the sides of the flasks.

It was desirable to make up the medium in the 10-liter bottles, which served as medium reservoirs. It was found that the phosphate buffer had to be sterilized separately so that precipitation would not occur. The buffer was then transferred to the 10-liter bottle, and the sterile constant-head siphon was affixed. Even with extreme care, cloudiness would sometimes appear in the medium reservoir denoting contamination. Then the somewhat laborious job of cleaning and sterilizing the unit had to take place.

When the unit reached the steady state, a sample had to be taken directly from it for the determination of the cell concentration. This would sometimes induce contamination, although a strenuous attempt was made to employ aseptic technique.

The actual counting of the two organisms in mixed culture at different dilution rates presented a problem since sometimes they were at widely different concentrations. The red organism would grow very rapidly and form an almost solid culture at each spot, masking the yellow organism. To partially counteract this effect, the 0.02 ml. spot was spread in a curved line, thus spreading out the organisms and allowing the slower yellow organism to show more clearly.

The author found that a standard curve plotting absorbancy versus number of cells/ml. was useful in determining the

number of organisms present in pure culture as a check on the plate count data and when a very large number of population density checks were desired. Experiments to obtain data for the standard curves were carried out exactly in the same manner as they were in the discontinuous flow studies and samples taken at several different absorbancies and counted (see Appendix, Figures 14 and 15).

In this study the author has reported the use of a highly controlled model waste purification system to study the predominance of one organism over the other. It is felt that this study is another basic step in eventual understanding of the causation of predominance in waste water purification. As has been previously stated, a better understanding of the causes of changes in predominance will allow more efficient waste water treatment to ensue.

CHAPTER V

SUMMARY AND CONCLUSIONS

The interaction of bacterial species leading to predominance of one specie over the other has been studied in both steady and non-steady state systems. From these studies it may be concluded:

1. Discontinuous flow studies may be used to determine certain kinetic constants necessary for predicting the growth patterns in continuous flow systems.

2. The substrate concentration does exert an effect on the exponential growth rate up to a critical value.

3. It would appear from these studies that cell concentration and the mode of predominance in mixed culture cannot be predicted in a quantitative sense on studies of the same organisms in pure culture. While it did prove possible, from the pure culture studies, to predict that S. marcescens would predominate in mixed culture, it was not possible to predict the steady state population density of each organism; nor was it possible to predict the more rapid "wash out" of the yellow organism in mixed as compared to pure culture.

4. The mechanism or mechanisms of the interaction of the species was not illuminated by this study.

CHAPTER VI

SUGGESTIONS FOR FUTURE WORK

In order to obtain a better understanding of predominance in various waste treatment processes, the following suggestions are offered:

1. Study two organisms isolated from waste water with approximately the same maximum growth rate in pure and mixed cultures and in continuous and discontinuous flow systems.

2. Study predominance patterns using a higher number of organisms isolated from waste water. This would create a much more complicated system and would be a step closer to the ultimate goal of predicting predominance in a heterogeneous population.

3. Do research on the mechanism of interaction with the aim of determining if the general or most probable cause in nature for the development of predominant species is simple competion for energy source or a form of antagonistic interaction.

4. Investigate the possible role of viruses in the determination of predominance in the heterogeneous population.

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APPENDIX

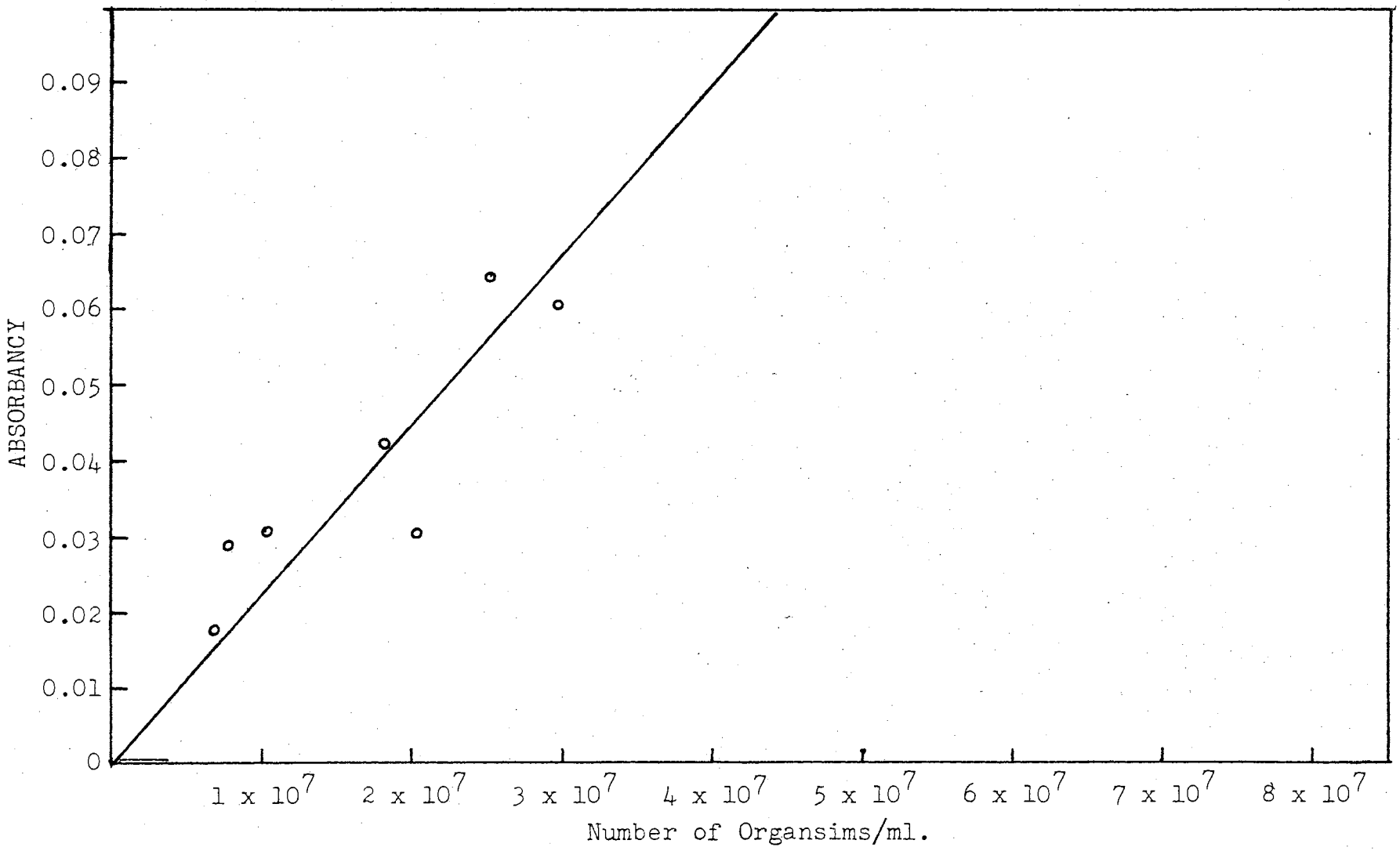

FIGURE 14 STANDARD CURVE FOR YELLOW ORGANISM

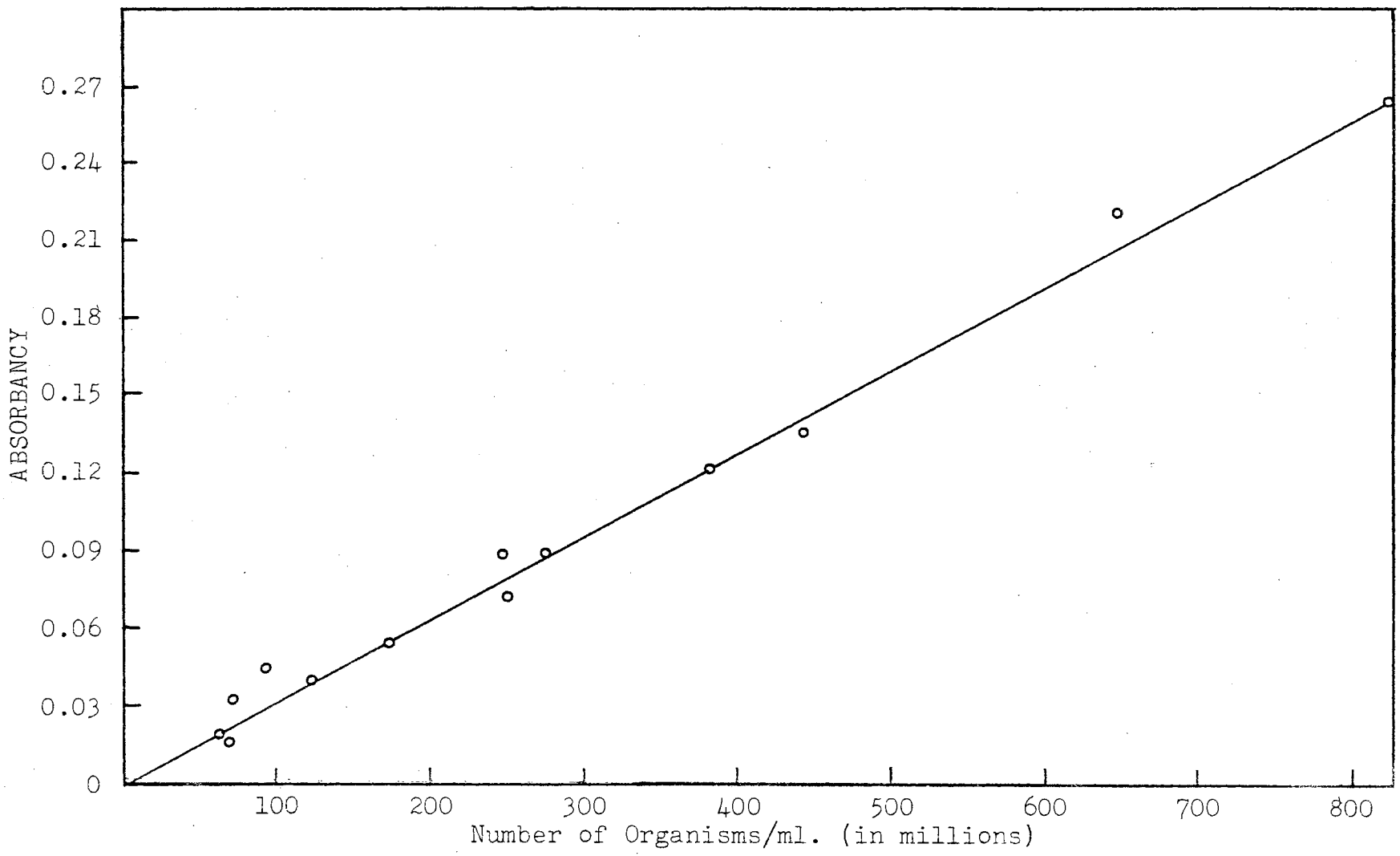

FIGURE 15 STANDARD CURVE FOR S. MARCESCENS

VITA

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Candidate for the Degree of

Master of Science

Thesis: PREDOMINANCE OF BACTERIAL SPECIES IN STEADY AND NON-STEADY STATE SYSTEMS

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Biographical:

Personal Data: Born in Laredo, Texas, January 14, 1940, the son of Alvin H. and Ruby Delle Leal.

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